Fatty-acylation target sequence in the ligand-binding domain of vertebrate steroid receptors demarcates evolution from estrogen-related receptors

J. Steroid Biochem. Mol. Biol., in press (2018)

Fatty-acylation target sequence in the ligand-binding domain of vertebrate steroid receptors demarcates evolution from estrogen-related receptors

Richard Lathe[a,*] and Douglas R. Houston[b,*]

[a] Division of Infection and Pathway Medicine, University of Edinburgh, Little France, Edinburgh EH16 4SB, UK

[b] School of Biological Sciences, University of Edinburgh, King's Buildings, Edinburgh EH9 3BF, UK

*Corresponding authors

Email addresses: richardlathe@ed.ac.uk (R. Lathe)

DouglasR.Houston@ed.ac.uk (D.R. Houston)

ABSTRACT

Present-day nuclear receptors (NRs) responding to adrenal and sex steroids are key regulators of reproduction and growth in mammals, and are thought to have evolved from an ancestral NR most closely related to extant estrogen-related receptors (ERRs). The molecular events (and ligands) that distinguish steroid-activated NRs (SRs) from their inferred ancestor, that gave rise to both the ERRs and SRs, remain unknown. We report that target sequences for fatty-acylation (palmitoylation) at a key cysteine residue (corresponding to Cys447 in human estrogen receptor ERα) in helix 8 of the ligand-binding domain accurately demarcate SRs from ERRs. Docking studies are consistent with the hypothesis that palmitate embeds into a key groove in the receptor surface. The implications of lipidation, and of potential alternative ligands for the key cysteine residue, for receptor function and the evolution of SRs are discussed.

Keywords: Estrogen; Estrogen-related receptor; Evolution; Fatty-acylation; Metalloestrogen; Palmitate; Steroid; Receptor

Highlights

The key structural changes underlying the transition from ERRs to vertebrate SRs are not known.

Extant SRs, exemplified by ERα, contain a site for cysteine fatty-acylation (principally palmitate) that is reported to promote both membrane association and receptor activity.

Comparative sequence analysis reveals that the fatty-acylation motif is present in SRs but is absent from all ERRs.

Molecular modeling indicates that palmitate locates to a groove in the surface of the ligand-binding domain, at a site that may modulate the 3D structure of the receptor.

Other potential interactions with the key cysteine residue include second-site steroid ligand and metalloestrogen binding.

Because the fatty-acylation sequence accurately demarcates SRs from ERRs, this modification may have played a role in the emergence of vertebrate receptors responding to steroids.

1. Introduction

Nuclear receptors (NRs) responding to adrenal and sex steroids in mammals orchestrate a spectrum of physiological processes including growth, immunity and inflammation, reproduction, stress, and water/salt homeostasis. All extant NRs comprise an N-terminal domain (NTD) and a DNA-binding domain (DBD), that are fused via a linker to a C-terminal ligand/hormone-binding domain [1]. The structure of the DBD is substantially conserved across all NRs, and we focus here on the ligand-binding domain and the ligands that bind to this module and activate transcription via binding of the receptor to specific DNA sequences.

The ancestral ligand for the first NRs in general could have been a 5-carbon terpenoid or a long-chain fatty acid [2;3], and studies on a remote species that contains only two NR polypeptides (*Amphimedon queenslandia*, a sponge) revealed that both NR1 and NR2 bind long-chain fatty acids. Interestingly, liganded NR1 activated transcription while liganded NR2 inhibited transcription [3]. Trichloplax, a simple multicellular animal at the base of metazoans, has been found to have orthologs of only four nuclear receptors – ERR, retinoid X receptor (RXR), COUP, and HNF4, but lacks conventional steroid receptors [4;5]. Early radiation of this group of receptors by gene duplication and mutation then generated other NRs.

Around 500 million years ago a sea-change took place in NR signaling with the evolution of the first receptors that responded to vertebrate steroids. This step was characterized by (i) the emergence of steroids, defined as molecules that lack all (or most of) the characteristic long hydrophobic sidechain of cholesterol – an oxidative reaction now catalyzed by the $P450^{scc}$ (sidechain cleavage; scc) enzyme – and (ii) the (co)evolution of the first vertebrate steroid NRs that that respond to sub-nanomolar concentrations of these more compact steroids.

The weight of evidence argues that vertebrate steroid-type NRs derive from an ancestral polypeptide that was most closely related to ERs [6-10], and elegant work has been done to reconstruct the ancestral receptor [10;11]. This ancestral ER-like receptor is presumed to have later diversified through gene duplication and mutation to generate the extant steroid receptors including ERα and ERα, as well as a steroid receptor that was the ancestor of the androgen, glucocorticoid, mineralocorticoid, and progesterone receptors (AR, GR, MR, PR) that respond to 3-ketosteroids. However, the key changes that precipitated the evolution of steroid-activated vertebrate receptors and the identities of their ancestral ligands (if any) remain unknown. A reconstructed ancestral SR appears to have had only low affinity for estradiol (E2), and potential ligands such as an aromatized cholesterol derivative have been suggested [10;11]; however, the full spectrum of other potential ligands has not yet been tested.

In the text the term AncSR is used to describe the common ancestor of the class of vertebrate receptors that first responded to steroid (or similar) ligands; this polypeptide corresponds to AncSR1 as defined by Thornton *et al.* [7;11] and to AncSR of Markov *et al.* [12]. The term AncERR is used to describe the earlier common ancestor that gave rise to the ERR class of receptors as well as to AncSR, and later to the vertebrate steroid receptors. Traditional nomenclature with Greek symbols (e.g., ERα) is employed to denote particular receptor types; however, to avoid the use of Greek symbols in computer analysis, in figures and when referring to a specific sequence entry the HUGO gene/protein guidelines (e.g., ESR1 for ERα) are followed.

In the following we focus on the structure and function of the ligand-binding domains of these receptors. We make a distinction between (i) the C-terminal module that responds to ligand, and (ii) the specific subregion, the ligand-binding pocket (LBP), that interacts directly with bound ligand.

1.1. The key changes accompanying ERR to SR evolution may be outside the LBP

Greschik and colleagues mutated key residues in the LBPs of the human ERRγ (ESRRG) and ERRα (ESRRA) to correspond to those found in ERα (ESR1), but this did not generate a high-affinity receptor responding to E2 [13;14]. Structural comparisons and sequence alignments of ERR ligand-binding domains versus ERα (e.g., [13;15;16], and supplementary data online) provide no evidence for major insertions or deletions. There is an insertion of eight residues between helices 8 and 9 in ERs that distinguishes ERs from ERRs (but this insertion is not present in AR, GR, MR, or PR), and also a three residue insertion between helices 9 and 10 could potentially be important (Figure S3 in the Supplementary Material Online).

We focused on helix 8 (H8) of the ligand-binding domain because (i) the H8–9 loop differs significantly between ERRα and ERα [14], (ii) mutation of Cys447 in H8 of ERα is reported to depress E2-mediated transcription activation by a large margin [17], and, importantly, as discussed below, (iii) this specific residue has been implicated in the covalent addition of long-chain fatty acids, the primary focus of this paper.

1.2. Covalent fatty-acylation of mammalian steroid receptors

Protein modification by covalent addition of long-chain fatty acids, principally palmitate, is conserved between yeast and mammals [18]. The modification is dynamic, and proteins can undergo multiple rounds of palmitoylation and depalmitoylation [19;20]. Human ERα contains a key cysteine residue within H8 of the ligand-binding domain, at a site distal from the hormone-binding site (LBP), which serves as an attachment site for a covalently bound palmitate chain [21]. A specific 9 amino acid motif surrounding the cysteine residue has been implicated in palmitoylation of human ERα, ERβ, PR, and AR [22]. All these NRs contain a sequence EFVCLKSII or close relative (the target cysteine is underlined); palmitoylation at this site (and consequent membrane binding) is thought to facilitate interactions with other membrane-bound proteins and promote fast non-genomic signaling ('membrane-initiated steroid signals' – MISS [1]).

Lipidation may also directly modulate receptor activity – mutation of the target residue Cys447 in human ERα, expressed in mammalian CHO cells, led to a 50-fold reduction in transactivation by E2, whereas mutation of other cysteine residues had no effect [17]. Moreover, in female mice, homozygous replacement of the corresponding cysteine residue in mouse ERα (Cys451) by Ala led to complete infertility and depressed E2-mediated gene expression ([23;24]; [1] for review). This evidence for the importance of the lipidation target site in H8 of ERα raises the question of whether this modification appeared early in the evolution of vertebrate steroid-activated NRs, and whether it could have played a role in ERR to ER evolution. We therefore studied the evolutionary history of palmitoylation in ERRs and ERs. We report that the lipidation target sequence accurately demarcates ERs and other SRs from ERRs, and we discuss the structural and functional implications for steroid receptor evolution.

2. Methods

2.1 Sequence and phylogenetic analysis

Sequences were drawn from publicly available databases including NCBI (https://www.ncbi.nlm.nih.gov/protein/), UniProt (http://www.uniprot.org/), and the Joint Genome Institute Genome Portal (https://genome.jgi.doe.gov/portal/). Sequence comparisons employed pBLAST searching (https://blast.ncbi.nlm.nih.gov/Blast.cgi); alignments were constructed using Clustal Omega [25] (https://www.ebi.ac.uk/Tools/msa/clustalo/) and displayed using mView (www.ebi.ac.uk/Tools/msa/mview/) using the color setting 'Any'. For phylogenetic analysis, trees were constructed using PhyML 3.0 [26] (http://www.atgc-montpellier.fr/phyml), and tree drawing employed FigTree (http://tree.bio.ed.ac.uk/software/figtree). Prediction of palmitoylation sites employed GPS-Lipid [27] (http://lipid.biocuckoo.org/webserver.php) and CSS-Palm [28] (http://csspalm.biocuckoo.org/online.php).

2.2. Structural modeling and molecular dynamics

Using the Build functions in PyMol 1.8.6.2 [29], the structure of the ERα ligand-binding domain (PDB 1A52; see [30;31]) was manually palmitoylated on Cys447, and a NAMD

topology was generated using the psfgen plugin of VMD 1.9.3 [32]. It was then solvated and neutralized by the addition of TIP3P water molecules [33] and Na^+ and Cl^- ions (to a concentration of 150 mM) to form a 108 Å × 108 Å × 108 Å simulation box. The full system comprised 115 689 atoms. NAMD 2.12 [34] was used for simulating this system, using the CHARMM36 force field [35] in a Langevin temperature and pressure controlled (NPT @ 310K) ensemble with periodic boundary conditions and particle-mesh Ewald electrostatics. Following energy minimization to remove van der Waals clashes within the system, a production run of 300 ns was performed. The Cα RMSD between starting structure and final frame of the simulation was 2.36 Å.

2.3. Docking studies

E2 was docked into the ERα ligand-binding domain (PDB 1A52) using Autodock. First, water molecules and other heteroatoms were removed from the structure, and the program PDB2PQR 2.1.1 [36] used to assign position-optimized hydrogen atoms, utilizing the additional PropKa2 algorithm [37] with a pH of 7.4 to predict protonation states. The MGLTools 1.5.6 [38] utility prepare_receptor4.py was used to assign Gasteiger charges to atoms. Hydrogen atoms were assigned to compound structures using OpenBabel 2.4.1 [39], utilizing the -p option to predict the protonation states of functional groups at pH 7.4. The MGLTools utility prepare_ligand4.py was used to assign Gasteiger charges and rotatable bonds. Autodock 4.2.6 [40] was used to automatically dock the compounds into the second ligand-binding site of the crystal structures. A grid box that encompassed the maximum dimensions of the ligand plus 12 Å in each direction was used. The starting translation and orientation of the ligand and the torsion angles of all rotatable bonds were set to random. The Autogrid grid point spacing was set at 0.2 Å. The Autodock parameter file specified 50 Lamarckian genetic algorithm runs, 15 000 000 energy evaluations, and a population size of 300.

3. Results

3.1. Lipidation sequences demarcate steroid receptors from precursors

We analyzed the ligand-binding domains of ERRs and steroid-type NRs in the vertebrate lineage (receptors and accessions are listed in Table S1 in the supplementary material online). As shown in Figure 1, none of the vertebrate ERR ligand-binding domains contain the target cysteine residue for palmitoylation, whereas (with some exceptions, discussed below) all vertebrate steroid NRs harbor a highly conserved sequence containing the key cysteine residue for lipidation. For example, in amphioxus (*Branchiostoma floridae*), that radiated shortly before the emergence of the vertebrates, the ER contains the sequence EYLCLKAIT, whereas the equivalent sequence in amphioxus ERR, EFVVLKAMA, lacks the key cysteine residue (Figure 1; detailed alignments are presented in Figures S2,S3). More divergent ERR-related sequences in fruitfly (*Drosophila melanogaster*), sea squirt (*Ciona intestinalis*), and sea urchin (*Strongylocentrotus purpuratus*) lack the key cysteine residue (Figure 2); this motif is also absent from all steroid receptor-related sequences studied from annelids, molluscs, and insects (see Discussion).

PhyML 3.0 was then used to assemble an evolutionary tree of the key region of ERRs and steroid-type NRs in the vertebrate lineage. As shown in Figure 3, this confirms the presence of a candidate palmitoylation sequence in the inferred ancestral steroid receptor (AncSR, equating to AncSR1 of Thornton *et al.* [11]), and in all present day ERs, as well as in AR, GR, and PR – but in none of the ERR-like receptors. This conclusion is reinforced by computer prediction of palmitoylation sites (Figure S4). Although the key cysteine is absent from MR, the tree demonstrates that representatives of antecedents to present-day MR, including AncSR, all contain candidate palmitoylation sequences, confirming that this motif was lost from MR in more recent evolution.

This analysis establishes a clear line of demarcation between the ancestral ERR and modern-day steroid receptors. The demarcation line is only blurred by MRs, where the lipidation sequence appears to have been lost later in evolution.

3.2. Molecular modeling – palmitate binds to a groove in the surface of ERα

To address the localization of the palmitate moiety with respect to receptor structure we performed molecular modeling. The ERα ligand-binding domain was manually palmitoylated on Cys447, a molecular dynamics production run of 300 ns was performed, and the results analyzed using the VMD molecular dynamics program. The simulation trajectory reveals two distinct conformations for the hydrophobic palmitoyl group, both of which are partially buried among hydrophobic residues of the protein (Figure 4). Throughout the first 38 ns the palmitoyl group showed a stable conformation within a tunnel leading to the E2 binding site (conformation 1, Figure 4A). After a short transition conformation in which the lipid chain is bent back upon itself, a second, more stable conformation was adopted, pointing away from the E2 binding site (conformation 2, Figure 4B), in which the fatty-acyl moiety resides within a groove in the surface of the receptor.

Because previous biochemical data indicate that E2 itself may bind to a second site within ERα in the vicinity of Cys447 [41], we performed molecular docking studies on the groove identified by palmitate binding. Autodock successfully reproduced the crystallographic binding mode of E2 to the ligand-binding site of ERα, with an RMSD from the crystallographic binding pose of 0.52 Å and a predicted ΔG of −10.1 kcal/mol (estimated K_d = 39.3 nM). Using the same protocol, E2 was docked into the putative second binding site, with the docking grid box centered on the palmitoyl chain in conformation 2 (Figure 4C). The ΔG of this second binding site was predicted by Autodock to be −7.59 kcal/mol (estimated K_d = 2.8 μM; ca 70-fold lower affinity), significantly weaker than the primary site. Key contact residues for palmitate and E2 (second-site) in the ERα ligand-binding domain are given in Table S6 and Figure S6.

3.2. Metal binding

In addition, it has been reported that Cys447 may participate in binding of physiological (Ca^{2+}) and environmental (e.g., Cd^{2+}) metals ('metalloestrogens') [42-44]. To address this we performed computational modeling, and a potential calcium binding site was identified sandwiched between helices H2, H8, and H9 of the ligand-binding domain of ERα. In Figure S7 the binding site is formed by the side chains of Cys447, Glu443, and Glu444 from H8, and

the backbone of H9. It is presumed that the site becomes solvent-accessible upon the same conformational changes that allow palmitoylation on 447; movement of H9 away from H8 to allow solvent access is also seen during the MD simulation.

4. Discussion

We report that the presence of a target sequence for lipidation in H8 accurately demarcates AncSR, ERs, and 3-ketosteroid receptors from the ERRs. This raises the question of whether lipidation sequences might have contributed to the emergence of AncSR from precursors.

Extant ERRs appear to be largely defective in ligand-dependent modulation of transcriptional activation, and are thought to be ligand-independent receptors (e.g., [45]). Mammalian ERRs (ESRRA, ESRRB, and ESRRG) do not bind E2; indeed, the LBPs of ESRRA and ESRRG [13;16] are significantly smaller than those found in ERs, potentially ruling out ligand-activated transcription by the ERRs. However, ligand-dependent transcriptional activation (or inhibition) of ERRs has been reported for both synthetic and natural ligands, but in all cases high, and ostensibly non-physiological, concentrations were required [46-50], with the possible exception of cholesterol as a potential natural ligand for ERRγ [50]. Although this situation could change if further studies uncover natural high-affinity ligands for the ERR group of receptors, the exquisite sensitivity of, for example, ERα for E2, with half-maximal activation at below 1 nM (possibly as low as ~50 pM), so far appears to be lacking in extant ERRs.

Together, these findings indicate that the ERRs inherently contain structural mechanisms for ligand-modulated transcription, but that the ligand-binding domain is constrained compared to the corresponding domain in steroid-type receptors, limiting physiological ligand-dependent activation of the receptor. The emergence of a fatty-acylation site in the ligand-binding domain of an ancestral ER could have accompanied changes in receptor structure that permitted high-affinity binding and transcription activation by E2, but the mechanism remains unclear. In the following we discuss (i) evolutionary antecedents, (ii) membrane localization as a potential contributor to the evolution of ERs, and (iii) the possibility of alternative ligands for the key cysteine residue.

4.1. Evolutionary antecedents

4.1.1. Amphioxus

Amphioxus denotes an early branch of the chordate lineage that radiated prior to the emergence of the vertebrates. Two vertebrate-like steroid receptors are present in *Branchiostoma floridae* (the Florida lancelet), and both BfER and BfSR contain the key H8 cysteine residue, whereas the BfERR lacks this residue (Figure 1). While BfER was not activated by any steroid tested (including E2, although binding to bisphenol A was reported) [51], BfSR was activated by both E2 and estrone at near-micromolar concentrations [52], a finding reiterated in the Japanese lancelet, *Branchiostoma belcheri* [53]. The lack of E2 activation of BfER is puzzling. Nonetheless, both BfSR and BfER harbor sequence features of both ERRs and SRs; for example, they retain the indel at 496–498 that is characteristic of

ERRs (Figure S3), and thus appear to represent an intermediate step between ERRs and vertebrate steroid-type receptors.

4.1.2. Insects, annelids, molluscs

Although our study focuses on the vertebrate lineage, several more distant members of the Bilateria [a group that includes both protosomes (e.g., nematodes, arthropods, and molluscs) and deuterostomes (e.g., vertebrates and echinoderms)], notably annelids, harbor both ERR-like receptors as well as a separate group of receptors, classifed as NR3D [10], that are reported to respond to E2 (see below). This prompted us to look wider.

In insects, where receptor activation by E2 has not been reported, BLAST searching with human ERα revealed that all close relatives (top 100 BLAST hits for the human ESR1 ligand-binding domain) lack a cysteine residue at the corresponding position, with the exception of some RXR-like receptors, a finding that might be fortuitous.

For molluscs (e.g., clams, oysters, snails, and octopi) the cysteine residue was also absent from polypeptides most closely related to human ERα (ESR1 ligand-binding domain BLAST search). NR3D receptors of molluscs have been reported to be constitutively active receptors [54], and thus resemble the ERRs.

In annelids (segmented worms), by contrast, there has been a report that NR3Ds from *Platynereis dumerilii* and *Capitella capitata* are capable of E2- (and estrone)-mediated transcription activation, but not by other steroids [55]. All close relatives (human ESR1 ligand-binding domain BLAST search) were found to lack the H8 cysteine residue. Phylogenetic analysis (PhyMl) indicated that two NR3D receptors (from *Mytilus edulis*, the blue mussel, and *Mytilus galloprovincialis*, the Mediterranean mussel) are closely related to the vertebrate ERR group of receptors, whereas the remaining NR3D polypeptides appeared to be more closely related to the vertebrate ER group, although constituting a distinct group(s) on their own (Figure S8). Nevertheless, phylogenies based on block sequence comparisons risk overlooking complexities such as subdomain motif exchanges (e.g., taking place by exon swapping or gene conversion). Moreover, the evolutionary ancestry of the annelid and mollusc receptors has been hotly debated (e.g., [10;56;57]), and the E2-responsive NR3D receptors of these species may possibly have an evolutionary origin that is distinct from vertebrate steroid-type receptors. Of note, the concentration of E2 necessary to activate the annelid NR3D receptors (ca 0.1 micromolar) contrasts with the sub-nanomolar concentrations of E2 that activate vertebrate ERs.

4.2. Palmitoylation and receptor function

The presence of a candidate lipidation sequence in vertebrate steroid-type receptors, but not in their evolutionary precursors represented by extant ERRs, invites consideration of the potential role of lipidation in receptor function. It is increasingly recognized that NRs can associate with membranes, where they can mediate fast non-genomic signaling ('MISS' [1]). Palmitoylation is thought to be an essential step in routing NRs to membranes. Palmitoylation is necessary for binding to caveolin (CAV1, itself a palmitoylated protein) that

in turn promotes shuttling of ERα to the membrane [22;58], and mutation of Cys447 in ERα abolished both membrane association and ERK activation [21;59].

However, palmitoylation is a dynamic modification, and cycles of palmitoylation/depalmitoylation are fundamental to signaling by some receptors (reviewed in [20]). In the case of the ER it has been suggested that E2 binding promotes removal of the covalently bound lipid, releasing the receptor from membranes and facilitating gene expression through receptor-mediated transactivation in the nucleus, thereby providing an additional ON–OFF toggle for ligand-dependent activation of transcription ([21;59]; reviewed in [1]). For these reasons, loss of membrane association *per se* is unlikely to explain the defects in E2-mediated gene expression seen in receptor mutants (or indeed in evolutionary antecedents) lacking the key cysteine residue.

Nevertheless, in a key paper, Reese and Katzenellenbogen [17] demonstrated that mutation of the palmitoylation site in ERα strongly impaired ligand-dependent transactivation, but that the block in the non-palmitoylated receptor could be overcome by a 50-fold excess of ligand. This indicates that there is a structural deficiency in the non-fatty-acylated receptor that may be overcome by either excess ligand or, plausibly, by the addition of a covalently attached long-chain fatty acid. Other studies concur that the key target cysteine residue in ERα plays a role in determining the higher-order structure of the ligand-binding domain and hormone-dependent activation of transcription [60;61]. In confirmation, mutation of the target cysteine residue *in vivo* in mouse ERα led to complete female infertility and downregulation of E2-responsive gene expression [23;24].

The finding that mutation of the key cysteine residue in ERα markedly reduces transcriptional activation (50-fold) without a major effect on E2 binding affinity (at best twofold), and that the defect can be overcome by a 50-fold excess of ligand [17], is nevertheless perplexing. Several explanations may be offered, such as deficits in coactivator binding (that was reported for this region of the ligand-binding domain in ERRα [14]) or in receptor dimerization [61], but this would not easily explain how the defect in ERα can be overcome by excess E2. By contrast, the observation is consistent with the possibility that two (or more) hydrophobic binding sites are present in the ligand-binding domain.

In support, it has been known for many decades that fatty acids can modulate NR function, including ER, GR, and PR, depending on their concentration, length, and degree of unsaturation, but do not compete with steroidal ligands targeting the LBP [62-66], pointing to a second interaction site that has not been firmly identified. In addition to fatty acids, to explain the curious pharmacologic behavior of antiestrogens, it has long been speculated that there may be one or more secondary binding sites on the estrogen receptor for steroidal ligands that do not compete with E2 for binding [67-73]; indeed, there may be several secondary sites [74].

Importantly, biochemical evidence indicates that the key target residue Cys447 of *E. coli*-produced ERα ligand-binding domain (lacking palmitoylation) immobilized on an E2 affinity column does not react with iodoacetic acid, whereas the free denatured protein reacted readily with iodoacetic acid at this site [41]; the authors argued that ligand binding in the vicinity of Cys447 (a site distal from the E2 LBP) restricts access to this specific residue.

Although the possibility that the affinity resin (sepharose) masks iodoacetic acid from interacting with Cys447 was not formally excluded, this would require hydrophilic contacts with a predominantly hydrophobic region of the ligand-binding domain. Our molecular dynamics and docking studies indicate that both palmitate and excess E2 may occupy a second hydrophobic pocket in the vicinity of H8 of the ERα ligand-binding domain. The target cysteine is within 4 Å of E2 (Table S6 and Figure S6), and E2 bound to this site could therefore mask Cys447 from chemical modification, as proposed [41].

The accumulated data thus suggest that covalent addition of a long-chain fatty acid to a hydrophobic site in the vicinity of H8 (or ligand excess) may lead to a change in receptor structure/interactions that facilitate transactivation mediated by ligand binding within the conventional LBP. The model could explain why some studies on Cys447 mutants of ERα did not detect significant downregulation of receptor activity at high (up to micromolar) ligand concentrations [21;59;61]. Nevertheless, the physiological relevance of E2 binding to this site is open to question. In addition to the supra-physiological ligand concentrations required, the 17β and 3β hydroxyl groups of E2 appear only to participate in binding to the second site via hydrogen bonding with the backbone (C3 hydroxyl to Leu310 oxygen, 2.7 Å; and C17 hydroxyl to Ile482 oxygen, 3.2 Å; supplementary data online): although (physiological) second-site ligands other than E2 are not excluded, E2 binding may take place fortuitously to a site built to accommodate a uniformly hydrophobic molecule such as palmitate.

By contrast, covalent addition of palmitate might radically change the dynamics of the interaction. Occupancy of the hydrophobic site close to H8 by covalently bound palmitate could stabilize a new conformation change or secondary interaction that promotes receptor activity in response to ligand binding to the conventional LBP. The acquisition of a lipidation target sequence in H8 of vertebrate NRs could thus have contributed to the emergence of steroid receptors capable of responding to sub-nanomolar concentrations of steroidal ligands. No crystal structure is so far available for NRs covalently modified by long-chain fatty acylation; however, if this technical obstacle can be overcome, it would be of great interest to explore to what extent this modification affects the overall structure of the ligand-binding domain. Further, it would be of great interest to determine whether addition of a palmitoylation sequence to ERRs, in conjunction with modifications to the LBP, might together generate a steroid-type receptor capable of responding to low (picomolar) concentrations of ligand. Equally, whether reintroduction of the missing cysteine motif into MR would have repercussions for ligand affinity or transcriptional activity.

4.3. Is there an alternative ligand for Cys447?

Although Cys447 is undoubtedly required for full receptor activity *in vivo*, and is a target for palmitoylation, there are some caveats. First, there is so far no evidence that palmitoylated receptors can enter the nucleus to activate transcription. Second, the inference that the key cysteine residue might undergo multiple cycles of palmitoylation and depalmitoylation is somewhat inconsistent with the fact that Cys447 is not well exposed at the surface (e.g., Figure 4), and might only be available to palmitoylation enzymes co-translationally. Third, in many cells only 5–10% of ER is palmitoylated and targeted to the cell membrane ([75] for review), and thus in these cells the key cysteine residue is largely free for other interactions.

We therefore considered whether the major population of ER (lacking palmitoylation) might have other binding partners.

Metal ions are candidate ligands for Cys447 because both physiological and environmental metals (metalloestrogens) are known to activate ER with high affinity (nM), and Cys447 has been specifically implicated. Environmental metals such as Cd^{2+} are known to exert adverse estrogenic effects across a range of vertebrate species by binding to the ligand-binding domain of ER [42-44;76]. Activation extends to physiological metals such as calcium that can modulate ER activity *in vivo*: extracellular Ca^{2+} increased the growth of MCF-7 cells via an ER-dependent mechanism, and chelation of Ca^{2+} blocked ERα activity [77]. Although not widely studied except in mammals, in addition to ERα and ERβ, heavy metals have been reported to interact with AR [78], GR [42;79;80], and PR [81].

In vertebrate ER the site(s) for metal binding in the LBD are distinct from the hormone-binding pocket, and several Cys, His, Asp, and Glu residues (including Cys447, Cys381, Glu523, His524, and Asp538) have been implicated as potential candidate interaction sites [42-44]. Significantly, mutation of Cys447 abolished metal-mediated receptor activation [42;43] indicating that, in addition to lipidation, this residue may be functionally liganded by physiological metals such as Ca^{2+}. In support, molecular modeling (Figure S7) indicates that Cys447, in conjunction with Glu443 and Glu44 in human ESR1, presents a potential binding site for Ca^{2+} binding. The two glutamate residues at 443 and 444 are substantially conserved throughout this group of receptors (Figure S3).

We note that Cys447 is not the only potential metal-reactive residue that differs between SRs and ERRs, and the evolution of SRs is accompanied not only by the emergence of Cys447 but also by loss of a cysteine residue corresponding to Thr347 in human ESR1 (Figure S3), suggestive of a switching process rather than merely the emergence of Cys447. No systematic work appears to have been done on metals and ERRs, nor on related receptors in more distant taxa including annelids and molluscs; further studies will be necessary to investigate whether, in addition to palmitoylation, metal binding might distinguish between steroid-type receptors and their evolutionary antecedents.

In sum, our finding that the key cysteine motif in H8 is present in vertebrate steroid receptors, but in none of their evolutionary antecedents, the ERRs, highlights the potential importance of the H8 region in the evolution of SRs, and concurs with several reports that this region plays a vital role in modulating the 3D structure of extant NRs [14;17;61;82]. Although it remains uncertain whether lipidation is the only ligand for the key cysteine residue, our findings corroborate the suggestion by Greschik *et al.* that this region of the protein could serve as an alternative to the LBP for the design of small-molecule drugs [14].

5. Conclusions

The molecular changes that led to the emergence of the first vertebrate steroid receptors are not yet understood. We report here a striking line of demarcation between vertebrate steroid receptors and ERRs, in that the former contain a conserved lipidation sequence that is entirely absent from the latter. This change could have accompanied the evolution of

steroid receptors from their inferred ancestor, AncERR, that give rise to both ERRs and steroid receptors.

There are many uncertainties: (i) although confirmed for ERα, ERβ, PR, and AR, it has not been formally demonstrated that this consensus sequence directs fatty-acylation across the spectrum of vertebrate NRs, and studies on the earliest representatives such as amphioxus ERR versus ER/SR or related species would be informative; and also (ii) it may not be assumed that the key cysteine residue (that is absent in ERR but present in steroid-type receptors) only guided lipidation, and it could be a target for an alternative physiological ligand such as metal ions (e.g., Ca^{2+}) or possibly fatty acids/sterols, or play another role so far unknown. Furthermore, (iii) evolution of SRs from AncERR undoubtedly proceeded through a progressive series of steps, and the emergence of the key cysteine residue is likely to represent only one of multiple modifications that led to modern-day vertebrate steroid-type receptors.

Nevertheless, given that the key H8 cysteine residue in the ligand-binding domain accurately demarcates extant steroid-type NRs from the ERRs, and that the lipid attachment site has been implicated in remodeling of receptor structure to facilitate transcriptional activation at low ligand concentrations, close scrutiny of the evolutionary and structural correlations between lipidation (and potentially metal binding) and NR evolution could shed light on the key changes that led to the emergence of steroid signaling in vertebrates.

Author contributions

R.L. performed sequence and phylogenetic analysis; D.H. performed molecular modeling studies. Both authors contributed to writing and editing of the MS.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgments

We would like to thank Michael E. Baker and John A. Katzenellenbogen for pivotal contributions to this work and stimulating discussions. S.J. Hillier and J.I. Mason are thanked for critical commentary. This work used the ARCHER UK National Supercomputing Service (http://www.archer.ac.uk).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/0000000000000000.

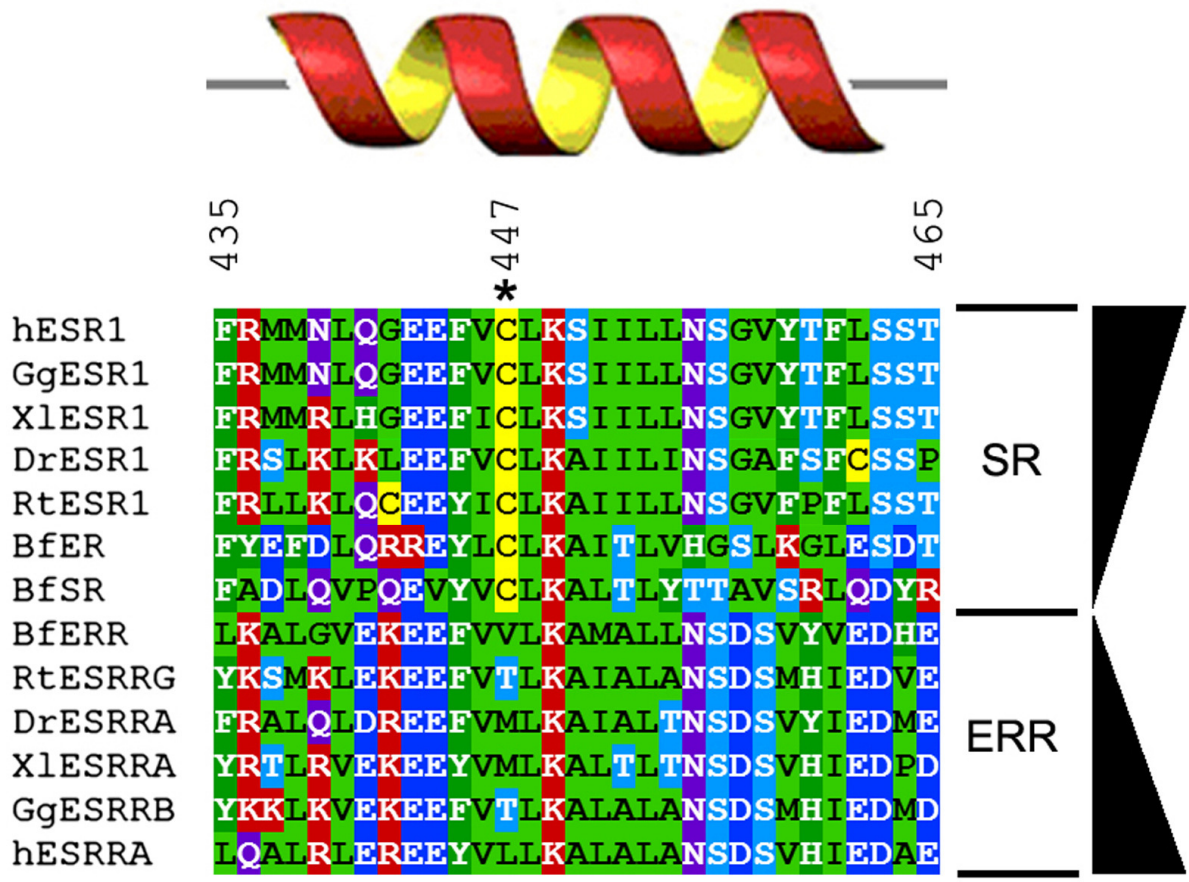

Figure 1. Color-coded alignment (simplified) of the central α-helix (H8) and flanking residues (435–465) in the ligand-binding domain of selected estrogen-related receptors (ERRs) and vertebrate receptors responding to steroids (SRs). Numbering corresponds to human ERα (ESR1). The cysteine residue that is absent from all ERRs, but present in SRs, is indicated (*, corresponding to Cys447 in human ERα). Extended alignments are presented in Figures S2 and S3. ERRs are presented in approximate evolutionary order, whereas SRs are presented in reverse order. Alignment display was performed using mView (www.ebi.ac.uk/Tools/msa/myview/) using default settings (color = 'Any'; yellow, Cys; red, Arg/Lys; dark blue, Asp/Glu; light blue, Ser/Thr; dark green, Phe/Tyr/Trp/His; purple, Gln/Asn; lighter green, other – Gly/Ala/Pro/Leu/Ile/Val/Met). Species names (prefixes): Bf, amphioxus, *Branchiostoma floridae*; Dr, zebrafish, *Danio rerio*; Gg, chicken, *Gallus gallus domesticus*; h, human; Rt, shark, *Rhincodon typus*; Xl, *Xenopus laevis*. Abbreviations: ER/ESR, estrogen receptor; ERR/ESRR, estrogen-related receptor; SR, steroid receptor.

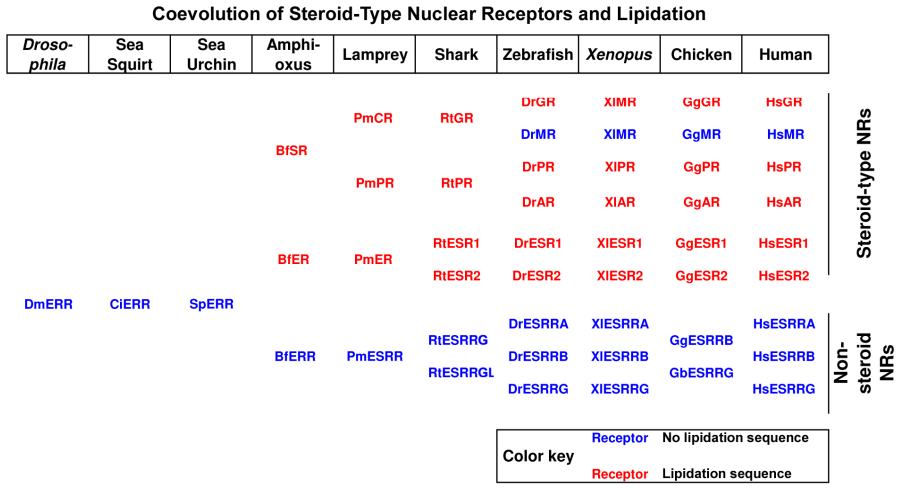

Figure 2. Palmitoylation sequences demarcate steroid-type nuclear receptors (red, containing the palmitoylation sequence) from precursor ERR (estrogen-related receptor) proteins (blue, that lack the key cysteine residue). Loss of the key cysteine residue in extant MRs appears to be a recent event. Species names (prefixes): Bf, amphioxus, *Branchiostoma floridae*; Ci, sea squirt, *Ciona intestinalis*; Dm, fruitfly, *Drosophila melanogaster*; Dr, zebrafish, *Danio rerio*; Gg, chicken, *Gallus gallus domesticus*; Hs, human; Pm, lamprey, *Petromyzon marinus*; Rt, shark, *Rhincodon typus*; Sp, sea urchin, *Strongylocentrotus purpuratus*; Xl, *Xenopus laevis*. Abbreviations. ESRR, vertebrate estrogen-related receptor; ER/ESR, estrogen receptor; AR, GR, MR, PR, androgen-, glucocorticoid-, mineralocorticoid-, and progesterone-type receptors, respectively; CR, lamprey corticosteroid-related receptor; SR, amphioxus (cortico)steroid-related receptor.

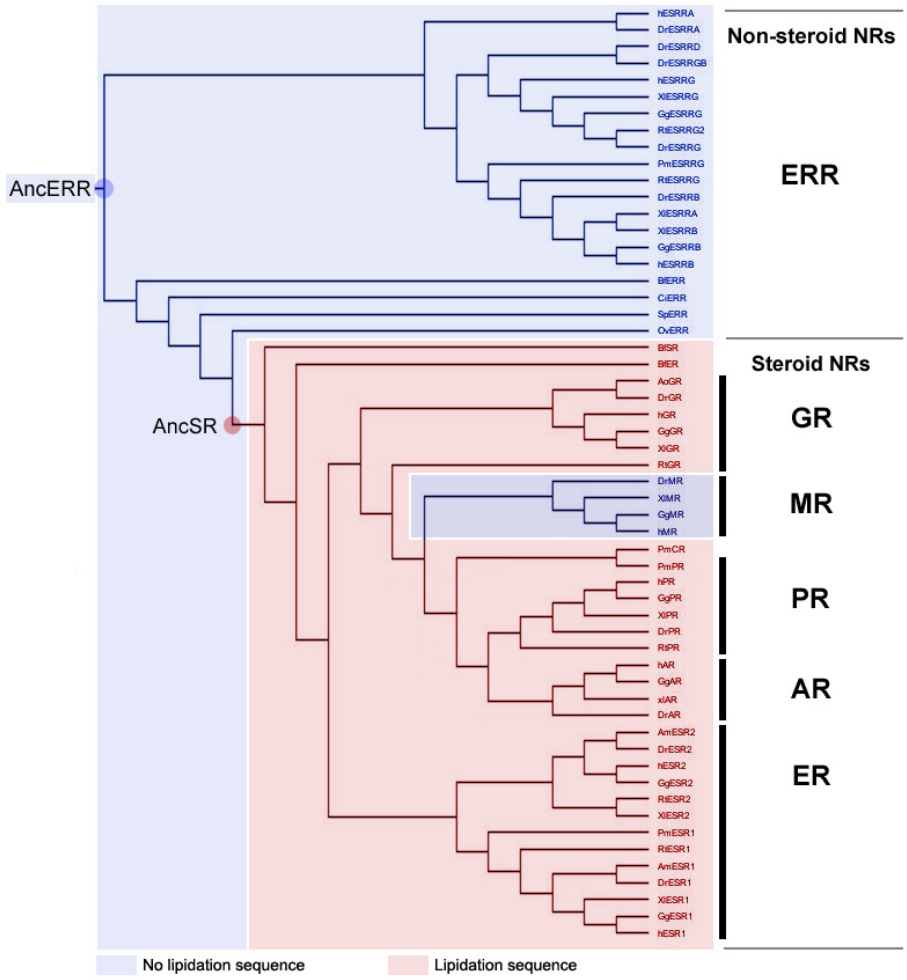

Figure 3. Phylogenetic analysis of the ligand-binding domains of estrogen-related (ERR) and steroid-type nuclear receptors (NRs). Loss of the lipidation target site is a recent event in the evolution of MRs (text for discussion). Sequences analyzed were 58 residues in length, from 26 residues before the key cysteine residue (or equivalent) to 31 residues following it; the identical tree was generated using the complete ligand-binding domains, 547 residues, of these receptors (sequences in Figure S3 in the supplementary material online). Phylogeny employed PhyML 3.0 (http://www.atgc-montpellier.fr/phyml); tree drawing was with FigTree (http://tree.bio.ed.ac.uk/software/figtree). Line length reflects the number of sequences (fewer ERRs in this analysis than steroid-type NRs), and not their evolutionary relatedness. Species names (prefixes): Am/Ao, clownfish, *Amphiprion melanopus/ocellaris*; Bf, amphioxus, *Branchiostoma floridae*; Dr, zebrafish, *Danio rerio*; Gg, chicken, *Gallus gallus domesticus*; h, human; Pm, lamprey, *Petromyzon marinus*; Rt, shark, *Rhincodon typus*; Xl, *Xenopus laevis*. PhyML identified ERR sequences from fruitfly, *Drosophila melanogaster*, sea squirt (*Ciona intestinalis*), and sea urchin (*Strongylocentrotus purpuratus*) as outliers (not presented) and these were therefore omitted from the tree. Abbreviations. AncERR, inferred ancestral ERR; AncSR, inferred ancestral steroid receptor (see Introduction); ESRR, vertebrate estrogen-related receptor; ER/ESR, estrogen receptor. AR, GR, MR, PR, androgen-, glucocorticoid-, mineralocorticoid-, and progesterone-type receptors, respectively; CR, lamprey corticosteroid-related receptor; SR, amphioxus (cortico)steroid-related receptor.

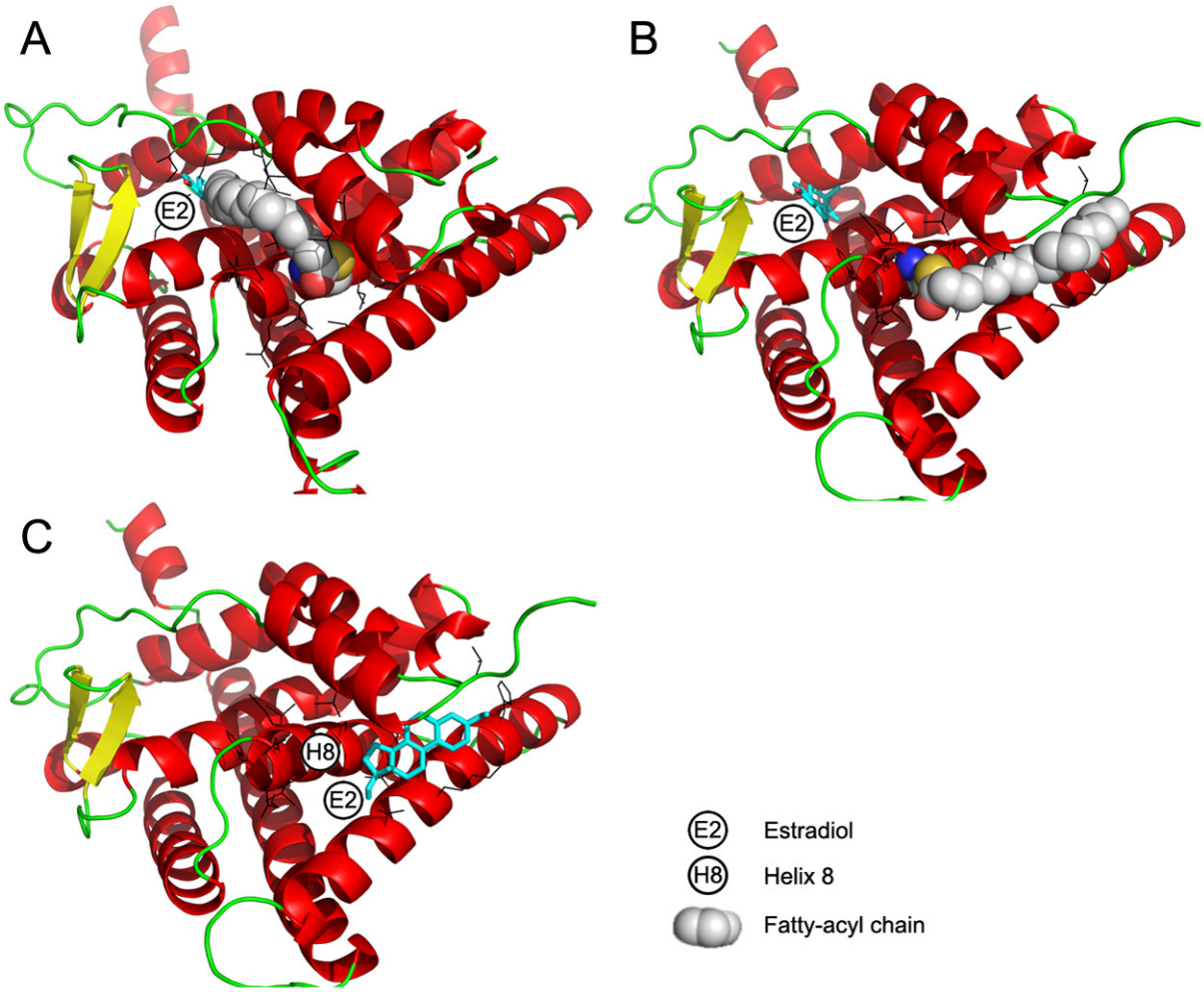

Figure 4. Molecular modeling, dynamics, and docking studies of the human ERα ligand-binding domain. (A) Manually built energy-minimized model of ERα palmitoylated (grey) on Cys447 in H8 (conformation 1). The protein secondary structure is shown as helices (red), sheets (yellow), and strands (green), the palmitoyl group as CPK-colored spheres, and sidechains within 4 Å of the lipid are shown as black lines. Estradiol (E2) is in light blue. (B) Conformation of the protein and palmitoyl group after 300 ns of molecular dynamics simulation (conformation 2). The palmitoyl group is now buried in a hydrophobic residue-lined groove, pointing away from the primary E2 binding site. (C) Result of docking E2 into the putative second binding pocket adjacent to helix 8. Larger versions of these figures are provided in Figure S5 in the supplementary material online.

References

[1] Arnal JF, Lenfant F, Metivier R, Flouriot G, Henrion D, Adlanmerini M, et al. Membrane and Nuclear Estrogen Receptor Alpha Actions: From Tissue Specificity to Medical Implications. Physiol Rev 97 (2017) 1045-1087.

[2] Moore DD. Diversity and unity in the nuclear hormone receptors: a terpenoid receptor superfamily. New Biol 2 (1990) 100-105.

[3] Bridgham JT, Eick GN, Larroux C, Deshpande K, Harms MJ, Gauthier ME, et al. Protein evolution by molecular tinkering: diversification of the nuclear receptor superfamily from a ligand-dependent ancestor. PLoS Biol 8 (2010) .

[4] Srivastava M, Begovic E, Chapman J, Putnam NH, Hellsten U, Kawashima T, et al. The Trichoplax genome and the nature of placozoans. Nature 454 (2008) 955-960.

[5] Baker ME. Trichoplax, the simplest known animal, contains an estrogen-related receptor but no estrogen receptor: Implications for estrogen receptor evolution. Biochem Biophys Res Commun 375 (2008) 623-627.

[6] Baker ME. Steroid receptor phylogeny and vertebrate origins. Molec Cell Endocrinol 135 (1997) 101-107.

[7] Thornton JW. Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions. Proc Natl Acad Sci U S A 98 (2001) 5671-5766.

[8] Markov GV, Laudet V. Origin and evolution of the ligand-binding ability of nuclear receptors. Mol Cell Endocrinol 334 (2011) 21-30.

[9] Baker ME, Nelson DR, Studer RA. Origin of the response to adrenal and sex steroids: Roles of promiscuity and co-evolution of enzymes and steroid receptors. J Steroid Biochem Mol Biol 151 (2015) 12-24.

[10] Markov GV, Gutierrez-Mazariegos J, Pitrat D, Billas IML, Bonneton F, Moras D, et al. Origin of an ancient hormone/receptor couple revealed by resurrection of an ancestral estrogen. Sci Adv 3 (2017) e1601778.

[11] Thornton JW, Need E, Crews D. Resurrecting the ancestral steroid receptor: ancient origin of estrogen signaling. Science 301 (2003) 1714-1717.

[12] Markov GV, Tavares R, Dauphin-Villemant C, Demeneix BA, Baker ME, Laudet V. Independent elaboration of steroid hormone signaling pathways in metazoans. Proc Natl Acad Sci U S A 106 (2009) 11913-11918.

[13] Greschik H, Wurtz JM, Sanglier S, Bourguet W, van DA, Moras D, et al. Structural and functional evidence for ligand-independent transcriptional activation by the estrogen-related receptor 3. Mol Cell 9 (2002) 303-313.

[14] Greschik H, Althage M, Flaig R, Sato Y, Chavant V, Peluso-Iltis C, et al. Communication between the ERRalpha homodimer interface and the PGC-1alpha binding surface via the helix 8-9 loop. J Biol Chem 283 (2008) 20220-20230.

[15] Baker ME, Chandsawangbhuwana C. Motif analysis of amphioxus, lamprey and invertebrate estrogen receptors: toward a better understanding of estrogen receptor evolution. Biochem Biophys Res Commun 371 (2008) 724-728.

[16] Kallen J, Schlaeppi JM, Bitsch F, Filipuzzi I, Schilb A, Riou V, et al. Evidence for ligand-independent transcriptional activation of the human estrogen-related receptor alpha (ERRalpha): crystal structure of ERRalpha ligand binding domain in complex with peroxisome proliferator-activated receptor coactivator-1alpha. J Biol Chem 279 (2004) 49330-49337.

[17] Reese JC, Katzenellenbogen BS. Mutagenesis of cysteines in the hormone binding domain of the human estrogen receptor. Alterations in binding and transcriptional activation by covalently and reversibly attaching ligands. J Biol Chem 266 (1991) 10880-10887.

[18] Linder ME, Deschenes RJ. Palmitoylation: policing protein stability and traffic. Nat Rev Mol Cell Biol 8 (2007) 74-84.

[19] Salaun C, Greaves J, Chamberlain LH. The intracellular dynamic of protein palmitoylation. J Cell Biol 191 (2010) 1229-1238.

[20] Sobocinska J, Roszcsenko-Jasinska P, Ciesielska A, Kwiatkowska K. Protein palmitoylation and its role in bacterial and viral infections. Front Immunol 8 (2018) 2003.

[21] Acconcia F, Ascenzi P, Bocedi A, Spisni E, Tomasi V, Trentalance A, et al. Palmitoylation-dependent estrogen receptor alpha membrane localization: regulation by 17beta-estradiol. Mol Biol Cell 16 (2005) 231-237.

[22] Pedram A, Razandi M, Sainson RC, Kim JK, Hughes CC, Levin ER. A conserved mechanism for steroid receptor translocation to the plasma membrane. J Biol Chem 282 (2007) 22278-22288.

[23] Adlanmerini M, Solinhac R, Abot A, Fabre A, Raymond-Letron I, Guihot AL, et al. Mutation of the palmitoylation site of estrogen receptor alpha in vivo reveals tissue-specific roles for membrane versus nuclear actions. Proc Natl Acad Sci U S A 111 (2014) E283-E290.

[24] Pedram A, Razandi M, Lewis M, Hammes S, Levin ER. Membrane-localized estrogen receptor alpha is required for normal organ development and function. Dev Cell 29 (2014) 482-490.

[25] Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol 7 (2011) 539.

[26] Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59 (2010) 307-321.

[27] Xie Y, Zheng Y, Li H, Luo X, He Z, Cao S, et al. GPS-Lipid: a robust tool for the prediction of multiple lipid modification sites. Sci Rep 6 (2016) 28249.

[28] Ren J, Wen L, Gao X, Jin C, Xue Y, Yao X. CSS-Palm 2.0: an updated software for palmitoylation sites prediction. Protein Eng Des Sel 21 (2008) 639-644.

[29] PyMol. The PyMOL molecular graphics system, version 2. Schrödinger LLC; 2015.

[30] Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, et al. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389 (1997) 753-758.

[31] Gangloff M, Ruff M, Eiler S, Duclaud S, Wurtz JM, Moras D. Crystal structure of a mutant hERalpha ligand-binding domain reveals key structural features for the mechanism of partial agonism. J Biol Chem 276 (2001) 15059-15065.

[32] Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. J Mol Graph 14 (1996) 33-38.

[33] Jorgensen WL, Chandrasekhar J, Madura JD, Impey RW, Klein NL. Comparison of simple potential functions for simulating liquid water. J Molec Graphics 14 (1983) 33-38.

[34] Phillips JC, Braun R, Wang W, Gumbart J, Tajkhorshid E, Villa E, et al. Scalable molecular dynamics with NAMD. J Comput Chem 26 (2005) 1781-1802.

[35] Huang J, Rauscher S, Nawrocki G, Ran T, Feig M, de Groot BL, et al. CHARMM36m: an improved force field for folded and intrinsically disordered proteins. Nat Methods 14 (2017) 71-73.

[36] Dolinsky TJ, Czodrowski P, Li H, Nielsen JE, Jensen JH, Klebe G, et al. PDB2PQR: expanding and upgrading automated preparation of biomolecular structures for molecular simulations. Nucleic Acids Res 35 (2007) W522-W525.

[37] Li H, Robertson AD, Jensen JH. Very fast empirical prediction and rationalization of protein pKa values. Proteins 61 (2005) 704-721.

[38] Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, et al. AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. J Comput Chem 30 (2009) 2785-2791.

[39] O'Boyle NM, Banck M, James CA, Morley C, Vandermeersch T, Hutchison GR. Open Babel: An open chemical toolbox. J Cheminform 3 (2011) 33.

[40] Forli S, Huey R, Pique ME, Sanner MF, Goodsell DS, Olson AJ. Computational protein-ligand docking and virtual drug screening with the AutoDock suite. Nat Protoc 11 (2016) 905-919.

[41] Hegy GB, Shackleton CH, Carlquist M, Bonn T, Engstrom O, Sjoholm P, et al. Carboxymethylation of the human estrogen receptor ligand-binding domain-estradiol complex: HPLC/ESMS peptide mapping shows that cysteine 447 does not react with iodoacetic acid. Steroids 61 (1996) 367-373.

[42] Stoica A, Katzenellenbogen BS, Martin MB. Activation of estrogen receptor-alpha by the heavy metal cadmium. Mol Endocrinol 14 (2000) 545-553.

[43] Martin MB, Reiter R, Pham T, Avellanet YR, Camara J, Lahm M, et al. Estrogen-like activity of metals in MCF-7 breast cancer cells. Endocrinology 144 (2003) 2425-2436.

[44] Nesatyy VJ, Rutishauser BV, Eggen RI, Suter MJ. Identification of the estrogen receptor Cd-binding sites by chemical modification. Analyst 130 (2005) 1087-1097.

[45] Tanida T, Matsuda KI, Yamada S, Hashimoto T, Kawata M. Estrogen-related Receptor beta Reduces the Subnuclear Mobility of Estrogen Receptor alpha and Suppresses Estrogen-dependent Cellular Function. J Biol Chem 290 (2015) 12332-12345.

[46] Tremblay GB, Kunath T, Bergeron D, Lapointe L, Champigny C, Bader JA, et al. Diethylstilbestrol regulates trophoblast stem cell differentiation as a ligand of orphan nuclear receptor ERR beta. Genes Dev 15 (2001) 833-838.

[47] Coward P, Lee D, Hull MV, Lehmann JM. 4-Hydroxytamoxifen binds to and deactivates the estrogen-related receptor gamma. Proc Natl Acad Sci U S A 98 (2001) 8880-8884.

[48] Zuercher WJ, Gaillard S, Orband-Miller LA, Chao EY, Shearer BG, Jones DG, et al. Identification and structure-activity relationship of phenolic acyl hydrazones as selective agonists for the estrogen-related orphan nuclear receptors ERRbeta and ERRgamma. J Med Chem 48 (2005) 3107-3109.

[49] Kim Y, Koh M, Kim DK, Choi HS, Park SB. Efficient discovery of selective small molecule agonists of estrogen-related receptor gamma using combinatorial approach. J Comb Chem 11 (2009) 928-937.

[50] Wei W, Schwaid AG, Wang X, Wang X, Chen S, Chu Q, et al. Ligand Activation of ERRalpha by Cholesterol Mediates Statin and Bisphosphonate Effects. Cell Metab 23 (2016) 479-491.

[51] Paris M, Pettersson K, Schubert M, Bertrand S, Pongratz I, Escriva H, et al. An amphioxus orthologue of the estrogen receptor that does not bind estradiol: insights into estrogen receptor evolution. BMC Evol Biol 8 (2008) 219.

[52] Bridgham JT, Brown JE, Rodriguez-Mari A, Catchen JM, Thornton JW. Evolution of a new function by degenerative mutation in cephalochordate steroid receptors. PLoS Genet 4 (2008) e1000191.

[53] Katsu Y, Kubokawa K, Urushitani H, Iguchi T. Estrogen-dependent transactivation of amphioxus steroid hormone receptor via both estrogen and androgen response elements. Endocrinology 151 (2010) 639-648.

[54] Keay J, Bridgham JT, Thornton JW. The Octopus vulgaris estrogen receptor is a constitutive transcriptional activator: evolutionary and functional implications. Endocrinology 147 (2006) 3861-3869.

[55] Keay J, Thornton JW. Hormone-activated estrogen receptors in annelid invertebrates: implications for evolution and endocrine disruption. Endocrinology 150 (2009) 1731-1738.

[56] Markov GV, Tavares R, Dauphin-Villemant C, Demeneix BA, Baker ME, Laudet V. Independent elaboration of steroid hormone signaling pathways in metazoans. Proc Natl Acad Sci U S A 106 (2009) 11913-11918.

[57] Eick GN, Thornton JW. Evolution of steroid receptors from an estrogen-sensitive ancestral receptor. Mol Cell Endocrinol 334 (2011) 31-38.

[58] Razandi M, Alton G, Pedram A, Ghonshani S, Webb P, Levin ER. Identification of a structural determinant necessary for the localization and function of estrogen receptor alpha at the plasma membrane. Mol Cell Biol 23 (2003) 1633-1646.

[59] Acconcia F, Ascenzi P, Fabozzi G, Visca P, Marino M. S-palmitoylation modulates human estrogen receptor-alpha functions. Biochem Biophys Res Commun 316 (2004) 878-883.

[60] Reese JC, Katzenellenbogen BS. Characterization of a temperature-sensitive mutation in the hormone binding domain of the human estrogen receptor. Studies in cell extracts and intact cells and their implications for hormone-dependent transcriptional activation. J Biol Chem 267 (1992) 9868-9873.

[61] Neff S, Sadowski C, Miksicek RJ. Mutational analysis of cysteine residues within the hormone-binding domain of the human estrogen receptor identifies mutants that are defective in both DNA-binding and subcellular distribution. Mol Endocrinol 8 (1994) 1215-1223.

[62] Hwang PL. Interaction of unsaturated fatty acids with anti-oestrogen-binding sites. Biochem J 243 (1987) 359-364.

[63] Kato J. Arachidonic acid as a possible modulator of estrogen, progestin, androgen, and glucocorticoid receptors in the central and peripheral tissues. J Steroid Biochem 34 (1989) 219-227.

[64] Benassayag C, Rigourd V, Mignot TM, Hassid J, Leroy MJ, Robert B, et al. Does high polyunsaturated free fatty acid level at the feto-maternal interface alter steroid hormone message during pregnancy? Prostaglandins Leukot Essent Fatty Acids 60 (1999) 393-399.

[65] Jansen MS, Nagel SC, Miranda PJ, Lobenhofer EK, Afshari CA, McDonnell DP. Short-chain fatty acids enhance nuclear receptor activity through mitogen-activated protein kinase activation and histone deacetylase inhibition. Proc Natl Acad Sci U S A 101 (2004) 7199-7204.

[66] Moutsatsou P, Papoutsi Z, Kassi E, Heldring N, Zhao C, Tsiapara A, et al. Fatty acids derived from royal jelly are modulators of estrogen receptor functions. PLoS One 5 (2010) e15594.

[67] Sutherland RL, Murphy LC, San FM, Green MD, Whybourne AM, Krozowski ZS. High-affinity anti-oestrogen binding site distinct from the oestrogen receptor. Nature 288 (1980) 273-275.

[68] Berthois Y, Pons M, Dussert C, Crastes de PA, Martin PM. Agonist-antagonist activity of anti-estrogens in the human breast cancer cell line MCF-7: an hypothesis for the interaction with a site distinct from the estrogen binding site. Mol Cell Endocrinol 99 (1994) 259-268.

[69] Jensen EV. Steroid hormones, receptors, and antagonists. Ann N Y Acad Sci 784 (1996) 1-17.

[70] Dudley MW, Sheeler CQ, Wang H, Khan S. Activation of the human estrogen receptor by the antiestrogens ICI 182,780 and tamoxifen in yeast genetic systems: implications for their mechanism of action. Proc Natl Acad Sci U S A 97 (2000) 3696-3701.

[71] Tyulmenkov VV, Klinge CM. Interaction of tetrahydrocrysene ketone with estrogen receptors alpha and beta indicates conformational differences in the receptor subtypes. Arch Biochem Biophys 381 (2000) 135-142.

[72] van Hoorn WP. Identification of a second binding site in the estrogen receptor. J Med Chem 45 (2002) 584-589.

[73] Wang Y, Chirgadze NY, Briggs SL, Khan S, Jensen EV, Burris TP. A second binding site for hydroxytamoxifen within the coactivator-binding groove of estrogen receptor beta. Proc Natl Acad Sci U S A 103 (2006) 9908-9911.

[74] Moore TW, Mayne CG, Katzenellenbogen JA. Minireview: Not picking pockets: nuclear receptor alternate-site modulators (NRAMs). Mol Endocrinol 24 (2010) 683-695.

[75] Levin ER. Plasma membrane estrogen receptors. Trends Endocrinol Metab 20 (2009) 477-482.

[76] Johnson MD, Kenney N, Stoica A, Hilakivi-Clarke L, Singh B, Chepko G, et al. Cadmium mimics the in vivo effects of estrogen in the uterus and mammary gland. Nat Med 9 (2003) 1081-1084.

[77] Divekar SD, Storchan GB, Sperle K, Veselik DJ, Johnson E, Dakshanamurthy S, et al. The role of calcium in the activation of estrogen receptor-alpha. Cancer Res 71 (2011) 1658-1668.

[78] Martin MB, Voeller HJ, Gelmann EP, Lu J, Stoica EG, Hebert EJ, et al. Role of cadmium in the regulation of AR gene expression and activity. Endocrinology 143 (2002) 263-275.

[79] Simons SS, Jr., Chakraborti PK, Cavanaugh AH. Arsenite and cadmium(II) as probes of glucocorticoid receptor structure and function. J Biol Chem 265 (1990) 1938-1945.

[80] Marketon JI, Sternberg EM. The glucocorticoid receptor: a revisited target for toxins. Toxins (Basel) 2 (2010) 1357-1380.

[81] Medici N, Minucci S, Nigro V, Abbondanza C, Armetta I, Molinari AM, et al. Metal binding sites of the estradiol receptor from calf uterus and their possible role in the regulation of receptor function. Biochemistry 28 (1989) 212-219.

[82] Deng Q, Waxse B, Riquelme D, Zhang J, Aguilera G. Helix 8 of the ligand binding domain of the glucocorticoid receptor (GR) is essential for ligand binding. Mol Cell Endocrinol 408 (2015) 23-32.